Immunological Profiles of Cancer Bearing Mice Fed a Black Raspberry Mediated Diet

Research Thesis

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By

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Abstract

Cancers of the pharynx and oral cavity account for 3% of new cancer cases in the United States each year. Approximately 90% of these cancers manifest as Head and Neck Squamous Cell Carcinoma. Recent preclinical trials have demonstrated the ability of black raspberries (BRBs) to inhibit oral carcinogenesis; however, the mechanisms behind this chemoprevention are not fully understood. Using the 4-nitroquinoline n-oxide (4NQO) carcinogen model of oral carcinogenesis, we induced head and neck cancers in wild type C57BL/6 mice. These mice were then fed either AIN-76A control diet or control diets supplemented with BRB, BRB-extract (BRB-E), protocatechuic acid (PCA, a constituent of BRB), or ellagic acid (EA, a BRB phytochemical). Mice were exposed to water containing 100 μg/mL 4NQO carcinogen for 16 weeks, after which they were exposed to clean drinking water for 8 weeks. Mice were then sacrificed, tumors and lesions of the tongue were counted, and the spleens and draining lymph nodes were collected. We isolated and plated single cell suspensions from spleens and lymph nodes of these mice, in the presence or absence of anti-CD3 antibodies and soluble CD28 antibodies. Sandwich cytokine enzyme-linked immunosorbent assays (ELISAs) were then performed on cell culture supernatants of these samples for the cytokines IL-2, IL-4, IL-6, IL-10, IL-17, TNF-α, and IFNγ. Dietary supplementation with BRB showed a significant decrease in IL-17 in the draining lymph nodes, as well as a significant increase in IL-2 in the spleen. These differences were also associated with a significant decrease in lesion multiplicity of mice fed the BRB supplemented diet. Our results demonstrate that, in the mouse 4NQO oral cancer model, the administration of dietary BRB inhibits oral carcinogenesis by mediating T-cell activation, proliferation, and differentiation. This provides an intriguing basis for follow up studies to further explore BRBs role in immune system regulation and oral carcinogenesis.

Introduction

Oral cancer accounts for about 3% of new cancer cases every year. It is estimated that in the US there are 54,010 new cases and 10,850 deaths every year due to oral cancer[1]. This equates to approximately 148 new cases and 30 deaths every day due to oral cancer. Globally, it is estimated that there are 377,713 new cases and 177,757 deaths annually due to oral cancer[2]. The vast majority of these oral cancers (90%) manifest as head and neck squamous cell carcinomas (HNSCC)[3]. The main risk factors of HNSCC are tobacco use, smokeless tobacco use, excess alcohol, and human-papilloma virus[4]. Avoidance of risk factors have only been slightly successful and despite modern advancements in treatment strategies for HNSCC, the survival rates of those affected have not improved in the past 30 years. As a result, new and effective approaches towards the treatment and prevention of HNSCC are needed.

Cancer chemoprevention strategies are used to slow carcinogenesis and tumorigenesis, as well as reduce the risk of recurrence and development of future cancer[5]. Diets rich in fruits and vegetables have been associated with a lower risk of developing oral cancer. Recent preclinical studies have demonstrated the ability of black raspberries (BRBs) to inhibit oral

carcinogenesis[6,7]. BRBs contain many active phytochemicals, such as protocatechuic acid (PCA) and ellagic acid (EA), which are thought to act to inhibit the development of cancer[7].

The 4-nitroquinoline n-oxide (4NQO) carcinogen model of oral carcinogenesis is one of the most common models of oral cancer chemoprevention. A recent study showed that in a rat 4NQO oral cancer model, dietary supplementation with BRBs inhibits oral carcinogenesis by the inhibition of pro-inflammatory and anti-apoptotic pathways[8]. However, the mechanisms behind BRB's ability of chemoprevention are not completely understood. We seek to fully explore and define these underlying mechanisms of oral cancer inhibition by BRBs and their phytochemicals, as well as confirm previous pre-clinical trial results demonstrating BRBs ability to inhibit oral carcinogenesis.

One particular area of interest is the effect of BRB on the production of various cytokines *in vivo*. To accomplish this, we used 4NQO to induce oral carcinogenesis in C57BL/7 mice which were then fed a normal diet, or a diet supplemented with BRBs or a BRB phytochemical. Single cell suspensions were taken from the draining lymph nodes (LN) and spleens of the mice and stimulated with anti-CD3 antibodies and soluble CD28 antibodies. Supernatants from these cultures were tested against seven different cytokines that are important in inflammation, immune system regulation and known to be associated with HNSCC and various immune pathways. Our results validate the ability of BRBs to inhibit oral carcinogenesis, as well as demonstrate BRB's role in the immune and inflammatory responses within the HNSCC microenvironment.

Materials and Methods

Animals

Male and Female wild type (WT) C57BL/6 mice, 7-8 weeks old, were used for these studies. The animals were housed in Ohio State University animal facilities in accordance with all state and federal guidelines established by University Laboratory Animal Resources (ULAR). The animal experiments were approved by the Institutional Animal Care and Use Committee (Protocol #2018A00000054) and Institutional Biosafety Committee of the Ohio State University.

Chemicals

The carcinogen 4NQO was purchased from Sigma-Aldrich (St. Lous, MO, USA; #N8141) and was stored at -20℃ wrapped in foil. Fresh 4NQO solutions (100 μg/mL in drinking water) were prepared twice weekly to be administered to the mice for 16 weeks. The BRBs used (*Rubus occidentalis* "Jewel variety") were obtained from the Stokes Berry Farm (Wilmington, OH, USA) and shipped frozen to Van Drunen Farms (Momence, IL, USA) for freeze drying. The BRB powder was stored at -20℃ until it was added into custom purified AIN-76A animal diet pellets at a 5% concentration.

Mouse Oral Carcinogenesis and Chemoprevention

A control sentinel group (Group 1, N = 10) received regular drinking water without the 4NQO carcinogen and was fed unmodified AIN-76A diet. The mice belonging to the experimental groups were randomized into five groups (Groups 2-6, N = 10 per group). These groups were administered 4NQO (100ug/ml) in their drinking water for 16 weeks, after which they were exposed to regular drinking water for 8 weeks. Group 2 (carcinogen control group) received the unmodified AIN-76A diet, Group 3 received AIN-76A diet containing 5% w/w BRB, Group 4 received AIN-76A diet containing 3% of an ethanolic BRB extract, Group 5 received AIN-76A diet containing 500 ppm PCA, and Group 6 received AIN-76A diet containing 0.4% EA. After the 16-week carcinogen exposure and 8-week regular water exposure (24-week protocol), the mice were sacrificed. The primary tumors, spleens, and draining lymph nodes of each mouse were harvested and gross lesions were counted, measured, categorized, and recorded.

T-Cell Stimulation and ELISA

Single cell suspensions of the spleens and draining lymph nodes (LN) were isolated and plated in the presence or absence of anti-CD3 antibodies and soluble CD28 antibodies for 72 hours. Cell supernatants from stimulated (+CD3) and non-stimulated (-CD3) cell samples were analyzed through Sandwich ELISAs for IL-2, IL-4, IL-6, IL-10, IL-17, TNF-α, and IFN-γ production. The plates were read at 405 nm using a Spectramax 190 plate reader and concentrations were determined by extrapolation from a standard curve generated by the Softmax Pro software based on each cytokine's initial standard concentration.

Statistics

Statistical analyses were performed using GraphPad Prism v9.0.0 (GraphPad Software, San Diego, CA). *t*-tests were used to determine statistically significant differences between groups and p-values ≤ 0.05 were considered significant.

Results

Total tumor and lesion multiplicity of cancer bearing mice from each experimental diet are shown in **Figure 1**. The concentrations of each cytokine with respect to each diet in the draining lymph nodes and spleens are shown schematically in **Figures 2-8**. Significant differences in concentrations between -CD3 and +CD3 samples, the sentinel group and cancer bearing control group, as well as differences between cancer bearing control group and each experimental diet, were determined via *t*-tests.

Total tumor and lesion multiplicity in cancer bearing mice

Figure 1A-B

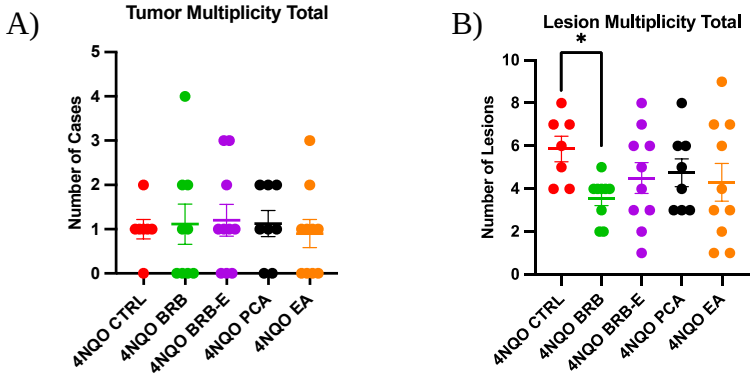

Figure 1) **a)** Total tumor multiplicity of cancer bearing mice fed experimental diets. **b)** Total lesion multiplicity of cancer bearing mice fed experimental diets. * significance determined by ANOVA test.

Levels of IL-2 in cell culture supernatants of LN and spleen stimulated cells

Figure 2A-L

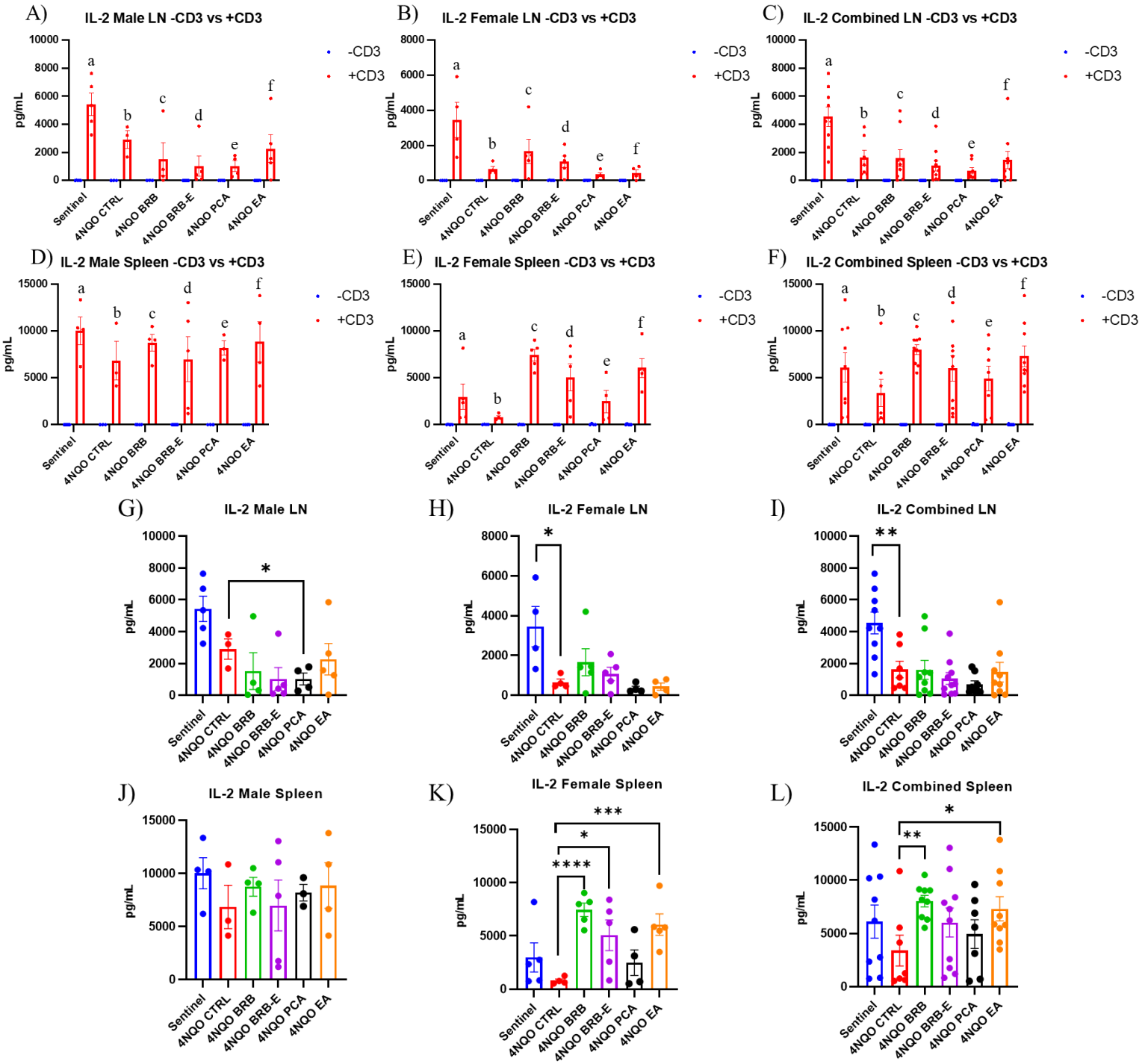

Figure 2)

Production of IL-2 in -CD3 stimulated samples compared to +CD3 stimulated samples in the **A)** male LN; a. **B)** female LN; a, c**. C)** combined LN; a, b, c, f. **D)** male spleen; a-f. **E)** female spleen; a, c, d, f. **F)** combined spleen; a-f. The letters noted, a-f, are considered significant, *p*-value ≤ 0.05. Significance was determined by multiple *t*-tests.

Production of IL-2 of only +CD3 simulated samples in the **G)** male LN, **H)** female LN, **I)** combined LN, **J)** male spleen, **K)** female spleen, **L)** combined spleen. * *p*-value ≤ 0.05; ** *p*-value ≤ 0.01; *** *p*-value ≤ 0.001; **** *p*-value ≤ 0.0001 between groups, as determined by *t*-test.

Levels of IL-4 in cell culture supernatants of LN and spleen stimulated cells

Figure 3A-L

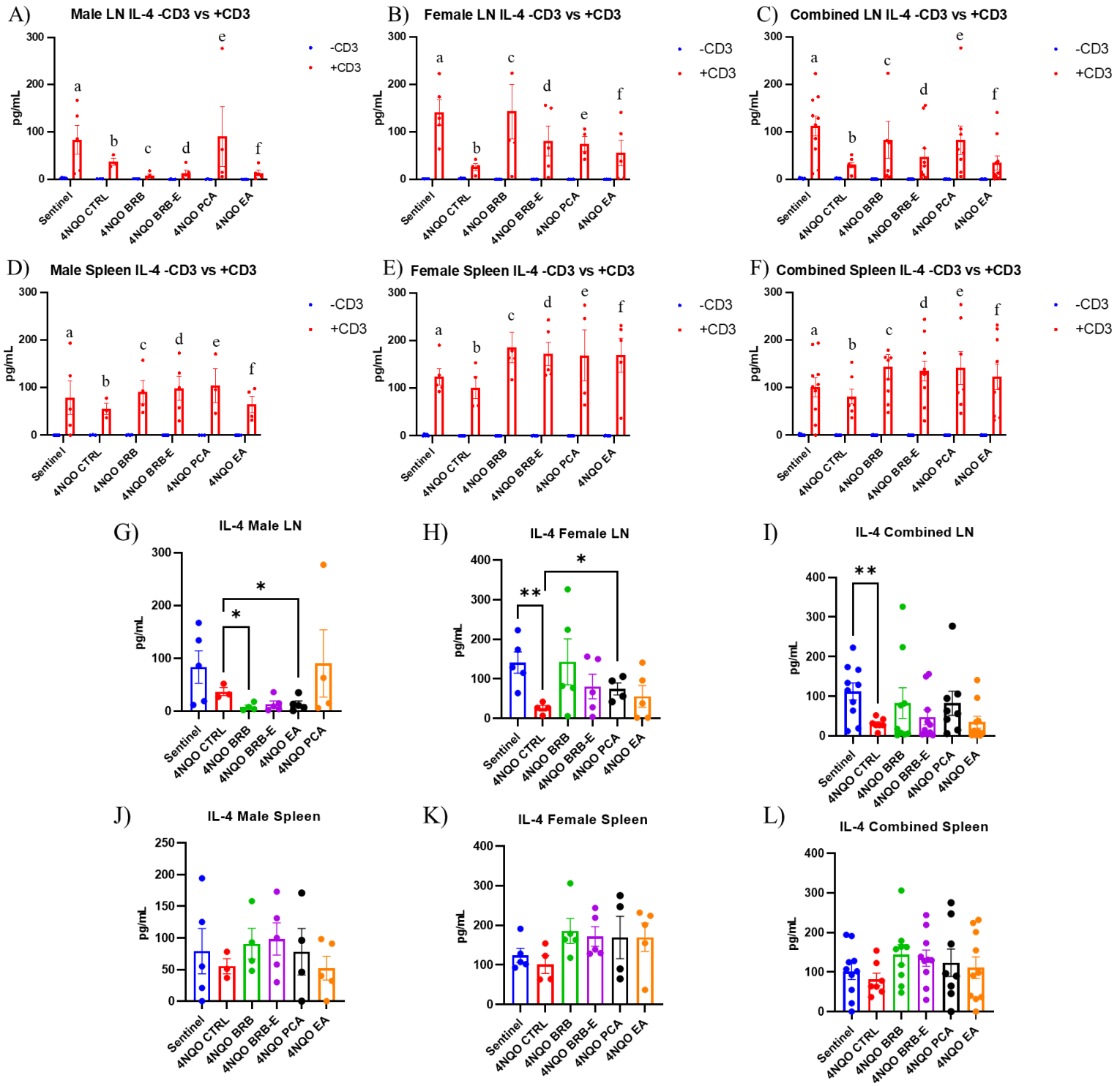

Figure 3)

Production of IL-4 in -CD3 stimulated samples compared to +CD3 stimulated samples in the **A)** male LN. **B)** female LN; a, c**. C)** combined LN; a, c, e. **D)** male spleen; a, c, d, e. **E)** female spleen; a-f. **F)** combined spleen; a-f. The letters noted, a-f, are considered significant, p-value ≤ 0.05. Significance was determined by multiple t-tests.

Production of IL-4 of only +CD3 simulated samples in the **G)** male LN, **H)** female LN, **I)** combined LN, **J)** male spleen, **K)** female spleen, **L)** combined spleen. * p-value ≤ 0.05; ** p-value ≤ 0.01; *** p-value ≤ 0.001; **** p-value ≤ 0.0001 between groups, as determined by t-test.

Levels of IL-6 in cell culture supernatants of LN and spleen stimulated cells

Figure 4A-L

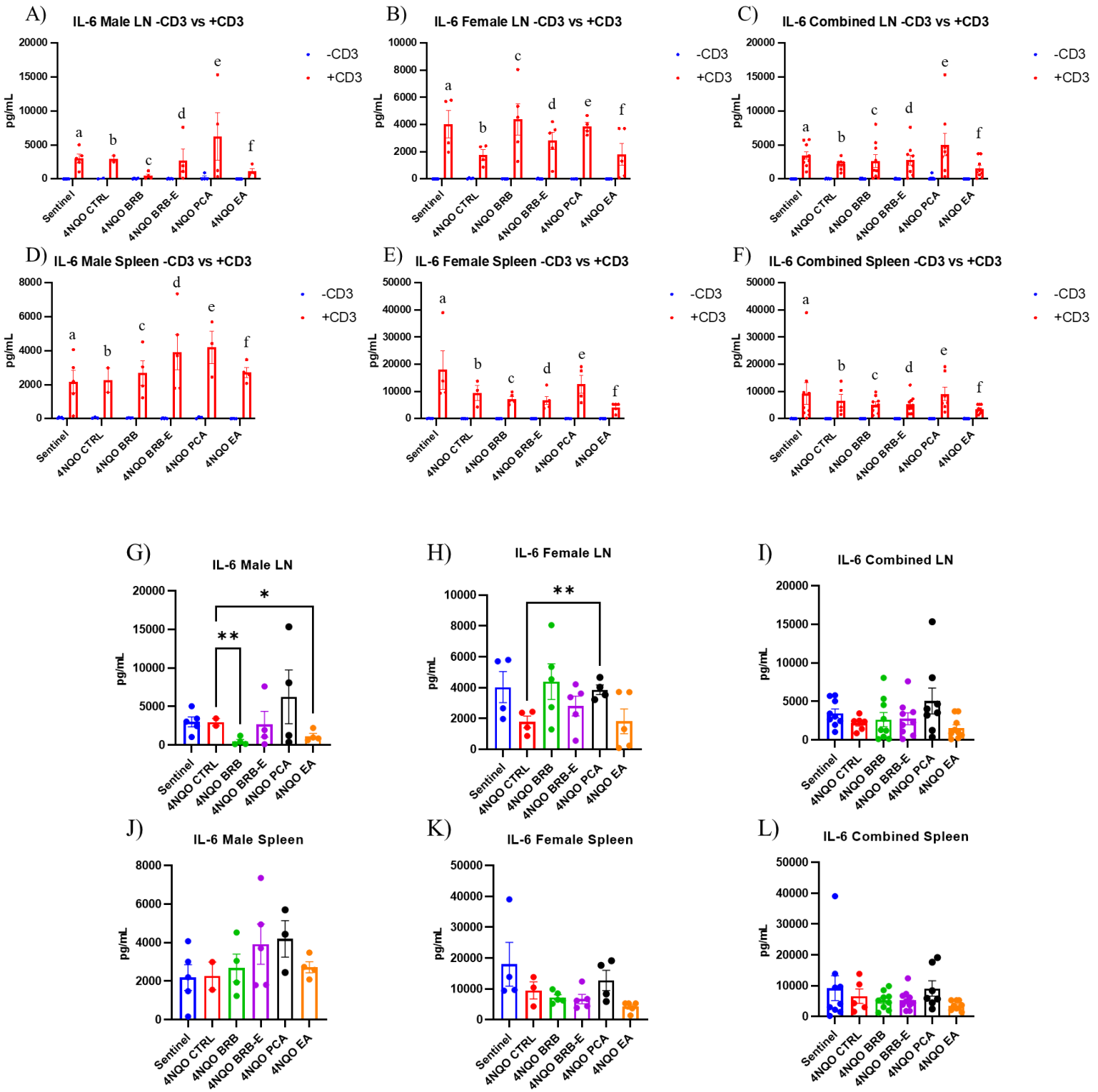

Figure 4)

Production of IL-6 in -CD3 stimulated samples compared to +CD3 stimulated samples in the **A)** male LN; e. **B)** female LN; a, c, d, e, f. **C)** combined LN; a, c, d, e. **D)** male spleen; a-f. **E)** female spleen; a, e. **F)** combined spleen; a, e. The letters noted, a-f, are considered significant, *p*-value ≤ 0.05. Significance was determined by multiple *t*-tests.

Production of IL-6 of only +CD3 simulated samples in the **G)** male LN, **H)** female LN, **I)** combined LN, **J)** male spleen, **K)** female spleen, **L)** combined spleen. * *p*-value ≤ 0.05; ** *p*-value ≤ 0.01; *** *p*-value ≤ 0.001; **** *p*-value ≤ 0.0001 between groups, as determined by *t*-test.

Levels of IL-10 in cell culture supernatants of LN and spleen stimulated cells

Figure 5A-L

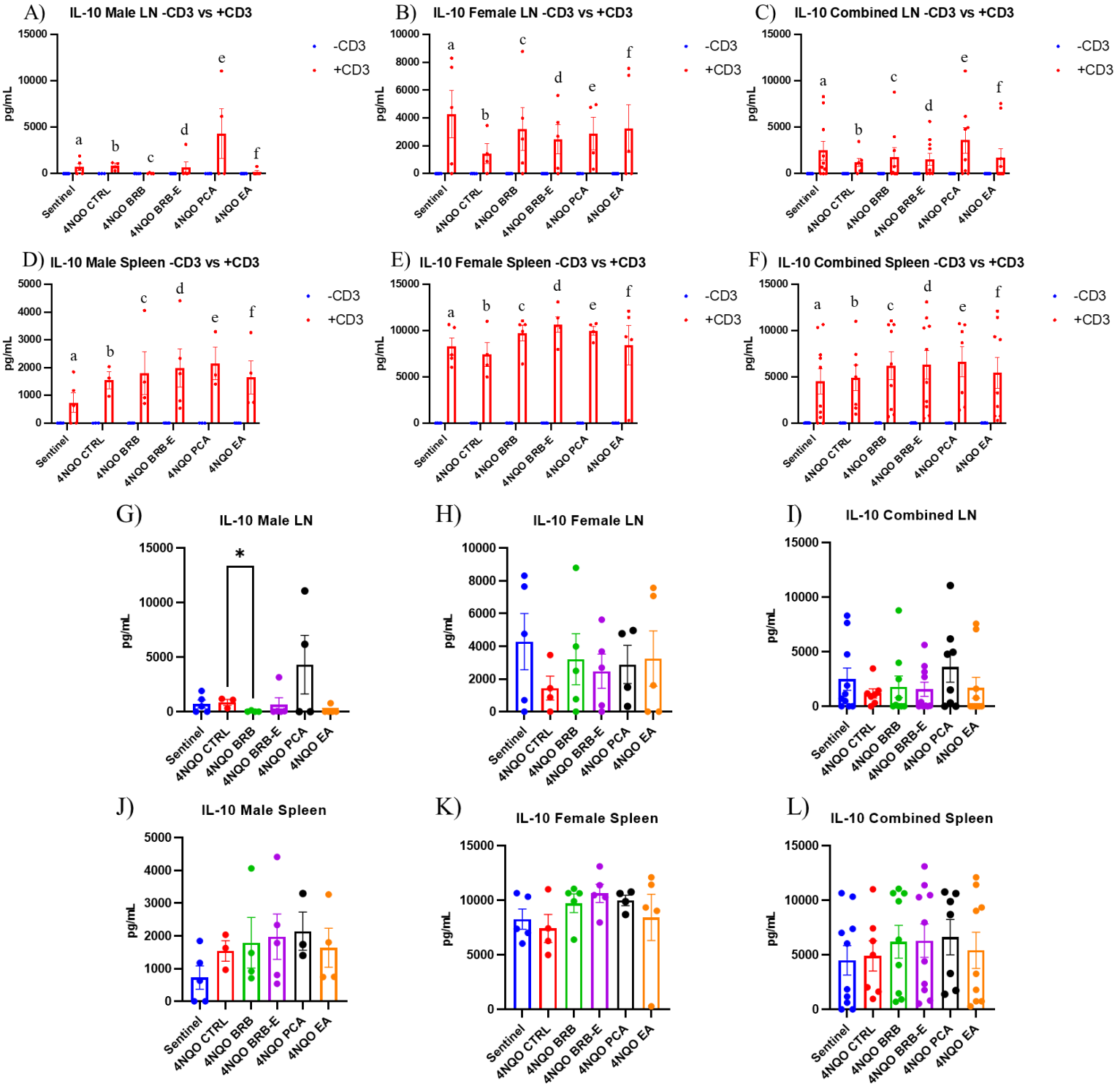

Figure 5)

Production of IL-10 in -CD3 stimulated samples compared to +CD3 stimulated samples in the **A)** male LN; e. **B)** female LN. **C)** combined LN; e. **D)** male spleen; c-f. **E)** female spleen; a-f. **F)** combined spleen; a-f. The letters noted, a-f, are considered significant, p-value ≤ 0.05. Significance was determined by multiple t-tests.

Production of IL-10 of only +CD3 simulated samples in the **G)** male LN, **H)** female LN, **I)** combined LN, **J)** male spleen, **K)** female spleen, **L)** combined spleen. * p-value ≤ 0.05; ** p-value ≤ 0.01; *** p-value ≤ 0.001; **** p-value ≤ 0.0001 between groups, as determined by t-test.

Levels of IL-17 in cell culture supernatants of LN and spleen stimulated cells

Figure 6A-L

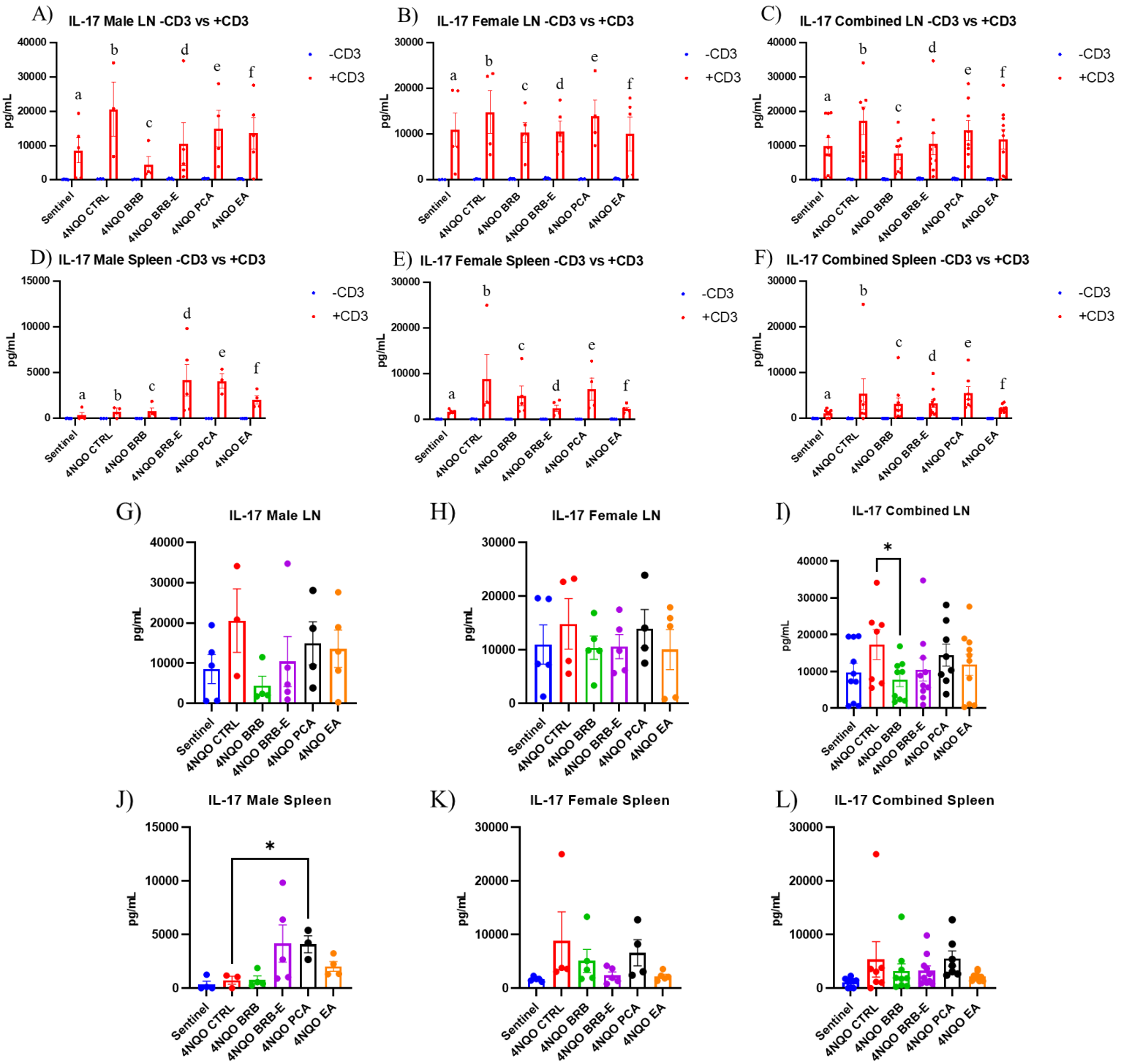

Figure 6)

Production of IL-17 in -CD3 stimulated samples compared to +CD3 stimulated samples in the **A)** male LN. **B)** female LN; a-f**. C)** combined LN; a-f. **D)** male spleen; d, e. **E)** female spleen; b. **F)** combined spleen; b, e. The letters noted, a-f, are considered significant, p-value ≤ 0.05. Significance was determined by multiple t-tests.

Production of IL-17 of only +CD3 simulated samples in the **G)** male LN, **H)** female LN, **I)** combined LN, **J)** male spleen, **K)** female spleen, **L)** combined spleen. * p-value ≤ 0.05; ** p-value ≤ 0.01; *** p-value ≤ 0.001; **** p-value ≤ 0.0001 between groups, as determined by t-test.

Levels of IFNγ in cell culture supernatants of LN and spleen stimulated cells

Figure 7A-L

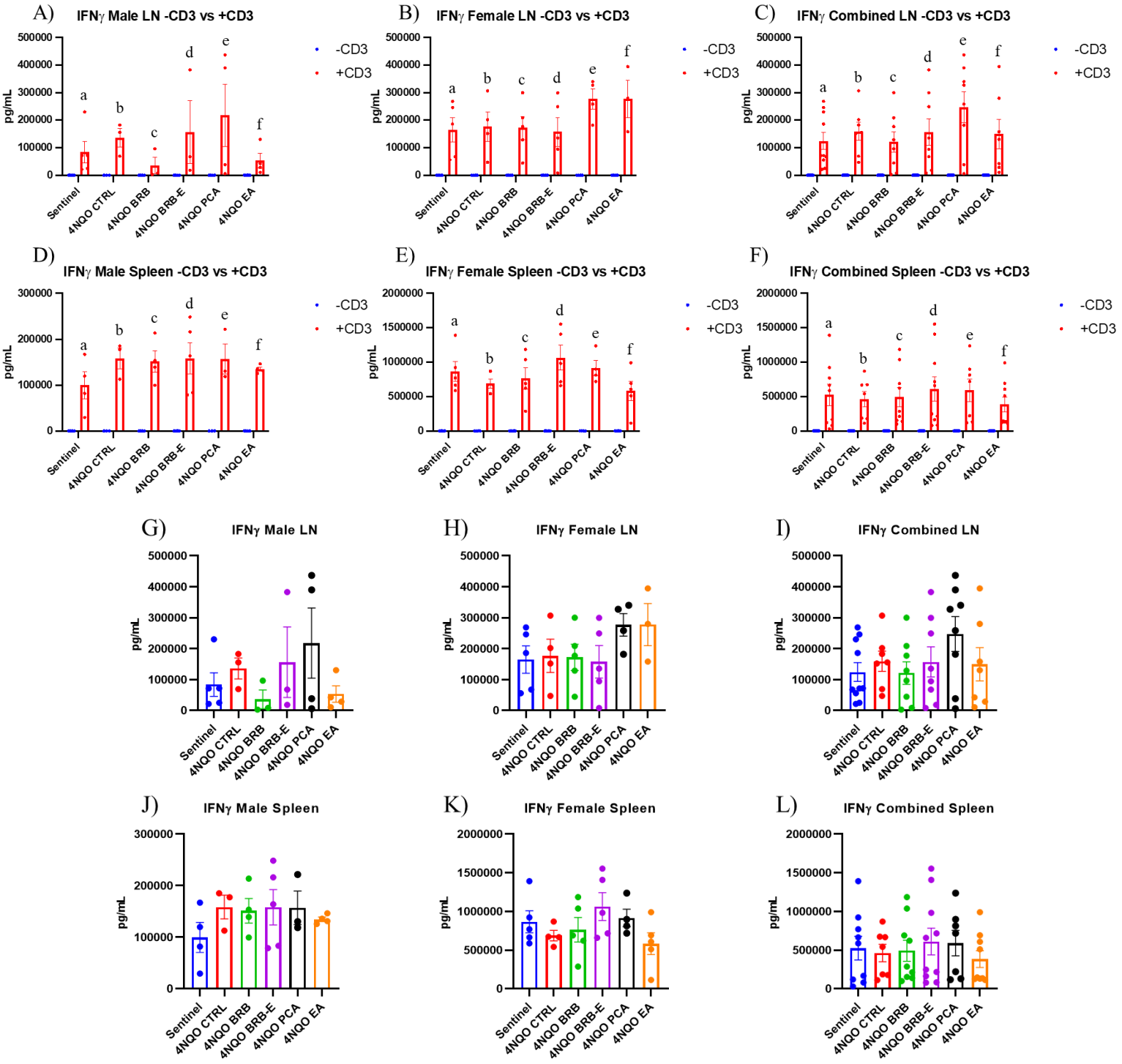

Figure 7)

Production of IFNγ in -CD3 stimulated samples compared to +CD3 stimulated samples in the **A)** male LN; e. **B)** female LN; a-f**. C)** combined LN; a-f. **D)** male spleen; a-f. **E)** female spleen; a-f. **F)** combined spleen; a-f. The letters noted, a-f, are considered significant, p-value ≤ 0.05. Significance was determined by multiple *t*-tests.

Production of IFNγ of only +CD3 simulated samples in the **G)** male LN, **H)** female LN, **I)** combined LN, **J)** male spleen, **K)** female spleen, **L)** combined spleen. * p-value ≤ 0.05; ** p-value ≤ 0.01; *** p-value ≤ 0.001; **** p-value ≤ 0.0001 between groups, as determined by *t*-test.

Levels of TNF-α in cell culture supernatants of LN and spleen stimulated cells

Figure 8A-L

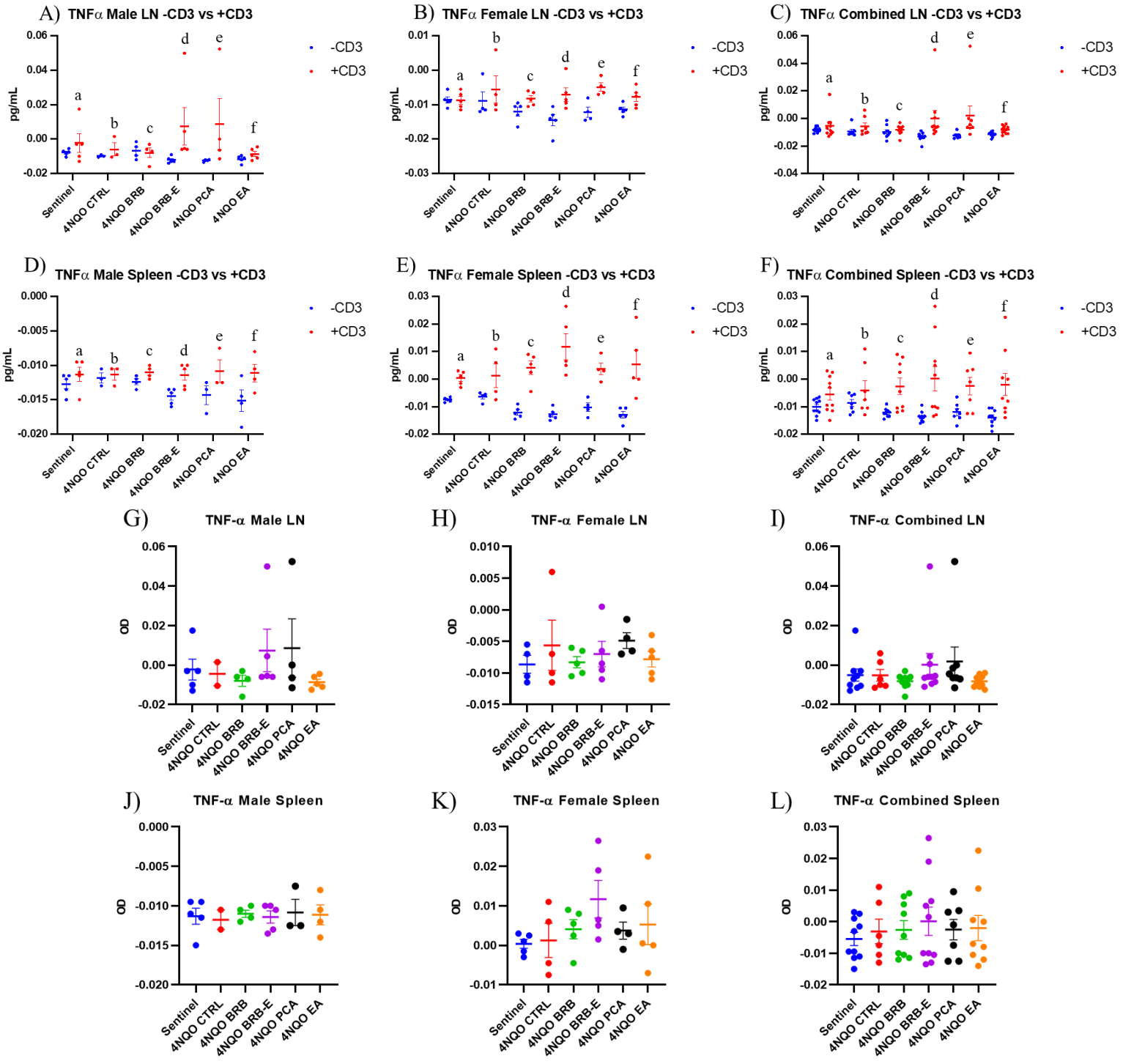

Figure 8)

Production of TNF-α in -CD3 stimulated samples compared to +CD3 stimulated samples in the **A)** male LN. **B)** female LN. **C)** combined LN; d, e. **D)** male spleen. **E)** female spleen; c-f. **F)** combined spleen; d, f. The letters noted, a-f, are considered significant, p-value ≤ 0.05. Significance was determined by multiple t-tests.

Production of TNF-α of only +CD3 simulated samples in the **G)** male LN, **H)** female LN, **I)** combined LN, **J)** male spleen, **K)** female spleen, **L)** combined spleen. * p-value ≤ 0.05; ** p-value ≤ 0.01; *** p-value ≤ 0.001; **** p-value ≤ 0.0001 between groups, as determined by t-test.

Initial tumor and lesion counts (Figure 1) show a significant decrease in lesion multiplicity of mice fed a BRB supplemented diet compared to the control diet (Figure 1B).

Sandwich ELISA reveals several changes in the levels of IL-2 between the experimental diets (Figure 2). We see a significant decrease in IL-2 in the LN of male mice fed the EA diet compared to the cancer bearing control (Figure 2G), as well as a significant decrease in the LN of female cancer bearing control mice compared to the sentinel group (Figure 2H). When the data is combined, a significant decrease in the LN of cancer bearing control compared to sentinel mice persists. Additionally, we see significant increases in IL-2 in the spleens of female mice fed the BRB diet, the BRB-E diet, and the EA diet, and significant increases in the combined data of mice fed the BRB diet and the EA diet.

For IL-4 (Figure 3), we see a significant decrease in male LN expression in the BRB diet and the EA diet (Figure 3G). Additionally, we see an increase in expression in the LN of females fed the PCA diet as well as in the cancer bearing control compared to the sentinel group (Figure 3H). When the groups are combined, a significant decrease of IL-4 in the LN of cancer bearing control compared to sentinel remains (Figure 3I). No significant differences of IL-4 were observed in the spleens of the mice; however in the combined group (Figure 3L), we see a decreasing trend in mice fed the BRB diet compared to the cancer bearing control ($p = 0.0753$).

A significant decrease of IL-6 was observed in the LN of male mice fed the BRB supplemented diet as well as the EA supplemented diet when compared to the control group (Figure 4G). A significant increase was observed in the LN of female mice fed the PCA supplemented diet (Figure 4H). However, these trends are not present in the total LN concentrations (Figure 4I). Additionally, no differences in the expression of IL-6 were seen in the spleens of any mice (Figure 4J-L).

We also see a significant reduction of IL-10 in the lymph nodes of the male mice fed a diet supplemented with BRB (Figure 5G), but no significant differences were found in the female LN or total LN concentrations (Figure 5H-I). Additionally, no differences in the expression of IL-10 were seen in the spleens of any mice (Figure 5J-L).

Our data for IL-7 shows no significant changes in the LN of males or females themselves (Figure 6G-H); however when they are combined, we see a significant decrease in the BRB mice compared to the cancer bearing control group. Additionally, we see a significant increase in IL-17 in the spleens of male mice fed the PCA mediated diet compared to the cancer bearing control group (Figure 6J), but no changes in the female spleens (Figure 6K) or in the combined spleens (Figure L).

Our results also show no changes in the levels of IFNγ (Figure 7G-L) or TNF-α (Figure 8G-L) in the LN or spleens of any of the mice fed experimental diets.

In summary, our results show a significant reduction in IL-17 production in the draining lymph nodes of mice fed the 5% BRB supplemented diet, as well as a significant increase in IL-2 in the spleens when compared to the cancer bearing control group. We also see a decreasing trend in the expression of IL-4 in the spleens of mice fed a BRB supplemented diet. Additionally, we see

a significant decrease in IL-4, IL-6, and IL-10 production in the draining lymph nodes of only male mice fed the 5% BRB supplemented diet when compared to our control, as well as a decreasing trend in IFNγ in these mice. No significant differences in the production of these cytokines were found when looking at the draining lymph nodes of the females.

Discussion

The tumor and lesion counts taken from the mice at terminal sacrifice demonstrated a significant reduction in lesion count in BRB-treated mice (Figure 1B). These initial results confirmed that BRB administration inhibits oral carcinogenesis; however, the effect of BRB on the immune systems of the mice was unknown.

The results from this study show that BRB supplemented diets had significant effects on increasing the levels of IL-2 (p=0.0053) in the spleens of oral cancer bearing mice. Originally discovered in 1976 as a T cell growth factor, IL-2 is a pleiotropic cytokine that is essential in the immune response [9,10]. IL-2 is produced mainly by $CD4^+$ T cells in response to antigen stimulation but is also produced in a lesser extent by $CD8^+$ T cells and other cell populations such as dendritic cells and thymic cells[11,12]. IL-2 promotes the differentiation of $CD4^+$ T cells to T helper 1 (Th1) and T helper 2 (Th2), as well as promoting the expansion of $CD8^+$ memory T cells and natural killer cells (NK)[13]. In addition to promoting Th1 and Th2 differentiation, IL-2 is known to inhibit differentiation of T helper 17 (Th17) cells as well as T follicular helper cells (Tfh)[14,15].

Taking this significant overexpression of IL-2 in the spleens of BRB-treated mice in conjunction with the significant decrease in lesion counts of these mice, our data suggests that in the mouse 4NQO model, BRB inhibits oral carcinogenesis by mediating the activation, proliferation, and differentiation of T cells. It has been hypothesized that oral cancer provides immunological impairment through imbalances in lymphocyte populations and their functions[16]. The activation, proliferation, and differentiation of these lymphocytes are known to be highly associated with levels of IL-2[11]. There have been many studies that demonstrate the ability of IL-2 to restore functions of impaired T cells[17]. Therefore, our results suggest that BRBs restore impaired T cell functions, as well as regulate lymphocyte populations in order to inhibit oral carcinogenesis.

Although our results suggest that BRBs contribute to the activation, proliferation, and differentiation of T cells during experimental oral carcinogenesis, the exact mechanism behind this impact remains unknown. However, the results from this study provide some interesting possibilities. In addition to seeing a significant increase in IL-2 in the spleens of mice fed 5% BRB, we also see a significant decrease in IL-17 in the draining lymph nodes. IL-17 is mainly produced by Th17 cells, and IL-2 is known to inhibit the differentiation of Th17 cells[14,18]. One possibility is that this potential decrease in Th17 cells in the BRB diet is associated with the inhibition of oral cancer. The other trend shown from the data is that along with the increase in IL-2 in the spleens of mice fed the 5% BRB diet, we also see an increasing trend in the expression of IL-4 (p=0.0753) in the spleens of these mice. IL-4 is essential for humoral immunity and is highly expressed in Th2 cells, in addition to be thought to play a role in immune

supression[19]. Therefore, the trend towards an increase in IL-4 expression, as well as a significant increase in IL-2 expression in mice fed the BRB supplemented diet, could demonstrate that BRB promotes the differentiation of Th2 cells. These trends provide interesting possibilities for follow up studies and analyses.

In conclusion, the results from this study demonstrate the ability of BRB to inhibit oral carcinogenesis, which is associated with regulating the T cell response. The significant increase in IL-2 in the spleens of mice fed a 5% BRB diet reveals the ability of BRB to restore the functions of impaired T cells and mediate T cell activation, proliferation, and differentiation. However, the exact mechanism of this ability is still unclear, and further studies should be conducted to elucidate these processes.

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